STUDIES ON THE DIGESTION OF RUMINANTS V. ENZYMES IN THE RUMEN OF GOAT AND CAMEL

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By

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It has been known that numerous micro-organisms found in the rumen of ruminants are playing an important role in digestive mechanism.[1] It may be safely conjectured that the relations between micro-organisms and the digestion of food might be weighty for the nutrition of ruminants.

We tried, therefore, to examine the enzymatic activity in the rumen contents containing micro-organisms. McAnally[2] and Umezu et al[3] reported in detail the enzymatic decomposition of cellulose contents but the other enzymes in the rumen have yet been scarcely studied.

First, various enzymes in the rumen contents of ruminant were tested in short period incubation and secondly further experiments were made by the method of long period incubation, for such enzymes not detected in the first experiment.

Experimental

Rumen contents of goat were taken by catheter and after centrifuging, (1,000 rotation in 1 min.) the upper clear solution was used as enzymatic preparate, containing living micro-organisms.

I. *Experiments of short period incubation*

Amylase, urease, proteinase, acid-phosphatase, alkaline-phosphatase, and lipase were ordinarily examined in short period incubation.

1. Amylase

Wohlgemuth's method was applied for determination of amylase. As enzyme solution, 1.0, 0.5, 0.25, 0.125, 0.063, 0.032, 0.016, 0.008, 0.004, 0.002 cc of the rumen contents were separately put into ten test tubes and water was added up to 1.0 cc in each test tube, except the first one, and then .2–5 cc of 0.1% soluble starch solution was added. After being incubated 30 min. at 37–38°C, they were diluted with salined water to 1 cc and added 1–2 drops of N/10 iodine solution. The pH of the medium was 7.0. According to the results the presence

of amylase was recognized though slightly, as shown in Table 1.

2. Urease

In the determination of urease, the modified method of Sumner was adopted. 3 g of urea and 6.8 g of Na_2HPO_4 and 2.8 g of KH_2PO_4 were dissolved in 100 cc distilled water. 20 cc of rumen contents was added to 20 cc of urea-phosphate solution. After it was incubated 20 min. at 37–38°C, 20 cc of 1N HCl was added to the solution. 10 cc of this solution was taken and NH_3 was determined by aeration method. The substrate solution without urea was used for the blank determination.

According to the results, no NH_3 evolved by enzymatic activity was detected.

3. Proteinase

Formol titration method was employed for the determination of amino-nitrogen which would be obtained by the proteinase activity. 5% of albumin solution was used as the substrate, and 10 cc of rumen contents was added to 10 cc of this solution. After it was incubated 60 min. at 37–38°C, 10 cc of formol solution was added, and was titrated with N/10 NaOH solution. Amino-nitrogen was determined by deducting the amount originally contained in the rumen contents and substrate from the titration readings.

As a results, no increase in amino-nitrogen was detected after incubation indicating a negative proteinase activity.

4. Acid-phosphatase

A modification of Bodansky's method was used for the measurement of acid-phosphatase. 0.5 g of β-sodium glycerophosphate and 30 cc of 1 M acetate buffer were made to 100 cc as the substrate buffer solution. 1 cc of the rumen contents was added to the 10 cc of this solution. After 60 min. incubation at 37–38°C, 10% trichloracetic acid was added to precipitate the protein and then was filtered. To 6 cc of the filtrate, 2 cc of molybdate solution (7.5 g/dlNa-molybdate was mixed with the same volume of 10 NH_2SO_4) and 2 cc of dilute solution of $SnCl_2$ (1 cc of 60g/dl solution of $SnCl_2$ was diluted to 400 cc) were added and blue colour developed were measured by the colorimeter. As the blank test, the inorganic phosphate in rumen contents and substrate was determined.

According to the results, the acid-phosphatase was present distinctly in the rumen contents, as shown in Table 1.

5. Alkaline-phosphatase

The method of the determination of alkaline-phosphatase is generally the same as that of acid-phosphatase. The only point of difference is that the veronal buffer solution was employed instead of acetic buffer solution, i.e. 0.5 mg β-sodium glycereophosphate and 0.424 g of veronal were dissolved in 100 cc as the substrate buffer solution.

According to the results, alkaline-phosphatase was detected in the rumen contents too.

6. Lipase

Modified method of Glick & King was used to detect the lipase. Olive oil was employed as substrate, 4 cc of rumen contents and 2 cc of substrate oil and 1 cc of 0.5×10^{-5} M/cc hexylresorcinol solution and 1 cc of phosphate buffer and 2 cc of distilled water were mixed as reaction medium. After incubation of the medium for 24 hours at 37–38°C, 25 cc of 90% alcohol was added to the reaction medium. Then fatty acid decomposed by enzyme was titrated with alkaline solution. Fatty acid in respective reagents and rumen contents employed was measured as the blank test. The results did not show the presence of the lipase in the rumen contents.

The experimental results obtained by the method mentioned above are shown in Table 1.

Table 1. Determination of various enzymes in rumen contents by the method of short period incubation which is generally employed.

	Method	Substrate	Condition of Reaction			Presence or absence	Contents
			Temp.	Time	pH		
Amylase	Wohlgemuth	starch	37-38°C	30min.	7.0	±	$d^{38°}_{30'}$ =2-5unit
Urease	Sumner	urea	〃	20	7.0	−	
Proteinase	Formol titration	albumin	〃	60	7.0	−	
Acid-phosphatase	Bodansky	β-sodium glycerophsphate	〃	60	5.0	+	11.73 mg/dl
Alkaline-phosphatase	〃	〃	〃	60	8.8	+	9.53 mg/dl
Lipase	Glick and King	olive oil	〃	24hrs.	8.0	−	

Summarizing all the above experimental results as shown in Table 1, urease, proteinase, and lipase were not found while amylase was observed slightly. However, alkaline-phosphatase and acid-phosphatase could be clearly discerned.

II. *Experiments of long period incubation*

Further experiments were carried out to test the possible activity of the enzymes by the method of long period incubation which was not discernible clearly in the first experiments.

In these experiments Omeliansky's medium[4] was used to dissolve substrate in the hope that a favorable condition might be produced for growth of the bacteria in rumen contents. Omeliansky's medium and experimental instruments were always sterilized in order to avoid contamination. Reaction medium was regulated to pH 6.6 by using phosphate buffer which was added

to Omeliansky's medium at 37–38°C.

A) Urease

Ammonium sulfate of Omeliansky's medium was replaced by urea. Thunberg's tube was employed for incubation. 2 cc of substrate and 2 cc of rumen contents in the main tube and 2 cc of N/50 H_2SO_4 solution in the side tube were added. Volatile NH_3 evolved during incubation was absorbed into N/50 H_2SO_4 solution in side tube and then titrated with alkaline solution and non-volatile NH_3 remained in the solution was measured by aeration method. Substrate medium without urea was used as control in all the expreiments. Measurements were made exactly following 2, 5, 10 days' incubation. Since

Table 2. Variation of NH_3 evolved by urease during long period incubation. (mg per 2cc of rumen contents)

a) In the case of 0.075% of urea.

Time of incubation (day)	Non-volatile NH_3	Volatile NH_3	Total NH_3	Decomposition ratio of urea	pH
2	0.15	0.02	0.17	10.0	7.4
5	0.16	0.09	0.25	14.7	7.4
10	0.17	0.18	0.35	20.0	7.6

b) In the case of 0.025% of urea.

Time of incubation (day)	Non-volatile NH_3	Volatile NH_3	Total NH_3	Decomposition ratio of urea	pH
2	0.12	0.02	0.14	24.6	7.2
5	0.14	0.03	0.17	29.7	7.2
10	0.11	0.04	0.15	29.1	7.2

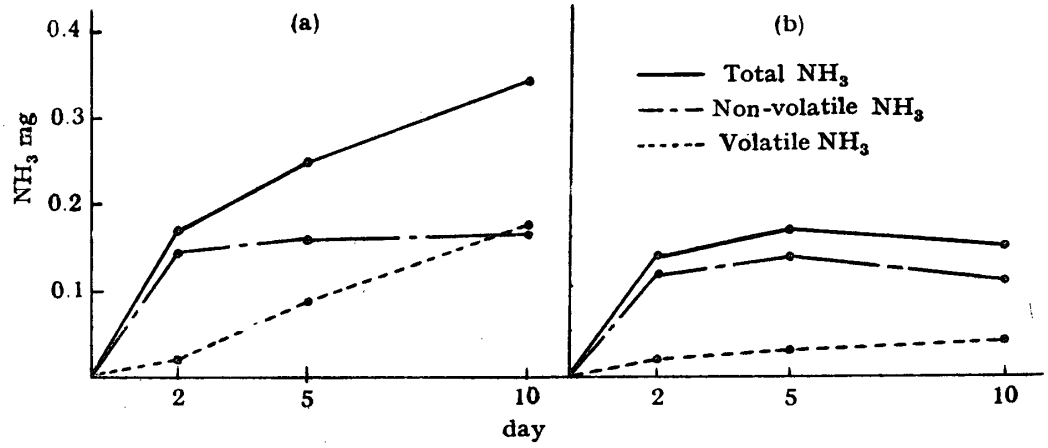


Fig. 1. Activity of urease in long period incubation
(a) 0.075% (b) 0.025%

it was already known in our laboratory that multiplication of bacteria in rumen contents is influenced by the concentration of urea[5], in the present work also we made some examination of two kinds of urea concentration. The results obtained are shown in Table 2 and Fig. 1.

According to our experimental results, NH_3 evolved by the urease activity could be clearly detected in either concentration. In the high concentration of 0.075% urea solution, the amount of volatile NH_3 gradually increased until the final determination after 2 days' incubation, while the increase of non-volatile NH_3 was very slow. Therefore, the increased amount of total NH_3 shown in the final period to be chiefly attributed to the increase of volatile NH_3. The pH value of reaction medium rose with the increase of NH_3 as shown in Table 2. When the low concentration of 0.025% urea was used, volatile NH_3 showed a tendency to increase slightly, while non-volatile NH_3 showed even some decrease after 5 days' incubation. Total NH_3 decreased in the final period contrary to the case of higher concentration. Comparing the amount of the total NH_3 decomposed by urease between the two reaction media of different concentrations of urea, it may be pointed out that NH_3 develops more in a higher concentration of urea than in a lower one. However, the decomposing ratio of urea was higher in the case of low concentration of urea than of high concentration.

Reaction medium without urea was used as the blank test for the experiments mentioned above. Furthermore, in order to confirm the function of urease the reaction medium without rumen contents or with boiled rumen contents also were examined as the control test. But in these cases no NH_3 could be found at all.

B) Proteinase

5% albumin solution was used as substrate and an equivalent amount of rumen contents was added to it. After this reaction medium was incubated for respective set periods, 1/2, 1, 3, 5, 7, 10 days, amino-N was determined by Van Slyke's method. The amounts of amino-N shown in Table 3 represent the values of differences between the amounts determined immediately before incubation and the amounts measured after incubation for respective durations.

Table 3. Amino-N decomposed by proteinase
(mg per 1 cc of rumen contents)

day	1/2	1	2	3	5	7	10
amino-N	0	0	0.05	0.18	0.4	0.48	0.54

According to the results, it was seen that protein decomposition took place already after 2 days' incubation producing amino-N. Afterwards, it gradually

increased to the maximum at the final test which showed 0.54 mg.

Recently Gray,[6] El-Shazly[7] reported that volatile fatty acids were produced by the protein decomposition in the rumen contents of goat. At any rate, it might be accepted that the presence of proteinase in the rumen contents was made clear by the present experiments as well as the report of Gray and El-Shazly.

C) Amylase

0.1% soluble starch solution was used as substrate. 1 cc of rumen contents was added to this solution and it was incubated.

Decomposed suger was measured by the method of Hagedorn-Jensen's method. Measurement was made four times namely, initial, 1 hr, 3 hrs, 24 hrs.

Since it was recognized that there was relatively abundant amylase in saliva, it was necessery to test whether the salivary amylase produced any effect on that of rumen contents or not. Similar method was applied for determination of salivary amylase in comparison with that of rumen contents. Sampling of saliva was made by the method of hypodermic injection of 0.5% pilocarpin hydrochloride solution.

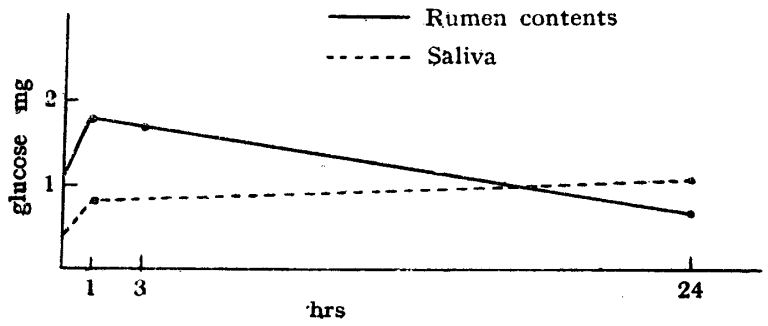
The experimental results are shown in Table 4 and Fig. 2.

Table 4. Decomposed sugar of starch by amylase activity.
(mg per 1 cc of rumen contents)

	Rumen contents				Saliva		
	Initial*	1 hr.	3 hrs.	24 hrs.	Initial*	1 hr.	24 hrs.
Sugar as glucose	1.1*	1.8	1.7	0.7	0.4**	0.8	1.1

* immediatly before incubation
* 1.1 = reduced sugar in the rumen contents 0.7
\+ reduced sugar in the substrate 0.4
** 0.4 = reduced sugar in the rumen contents 0
\+ reduced sugar in the substrate 0.4

Fig. 2. Amounts of sugar decomposed by rumen contents and saliva.



According to the results, an increase of sugar contents by the amylase activity was detected after 1 hr's incubation in the case of rumen contents. But a decrease was observed after 3 hrs and further decrease after 24 hrs.

The amylase activity of saliva was ostensibly much lower than that in rumen contents. This phenomenon of decreased sugar in the later parts of the incubation period may be explained by the assumption that the produced sugar was utilized by the micro-organisms in the rumen contents. This fact agrees precisely with the results given in our previous report[8].

Yamafuji et al[9] reported that amylase was present in the rumen contents of cow. In our present work, amylase was detected in the rumen contents of goat by this method, although it was not clearly found by the Wohlgemuth's method. It was also detected by Wohlgemuth's method in the rumen contents of a camel which was burnt to death in a fire in the Zoo.

In any case, it may safely be said that the amylase is present in the rumen contents of ruminants.

Discussion

According to the results of our experiments conducted concerning the activities of various enzymes in the rumen contents of goat, phosphatase activity alone was found clearly by the method of short period incubation, a method generally used in the study of enzymatic activity.

The presence of the phosphatase in the rumen contents is thought to be related to the facts that phosphatase can be detected also in the rumen mucosa by a similar method[10] and that the possibility of inorganic phosphate absorption in the rumen is reported.[11]

Then, the enzymes which were unable to be detected by the usual method of short period incubation — urease, proteinase — will be discussed below.

Since urea is an important material for the nitrogen nutrition of ruminant, the presence or absence of urease in the rumen contents is a highly interesting problem. The studies on the utilization of urea were carried out by Pearsson and Smith.[12] In these studies the artificial rumen technique was employed. According to these results, it was found by them that urea were converted into ammonia in a relatively short period. They reported that urease must be elaborated by micro-organisms in the rumen contents.

Iwata et al[13] reported that the rumen contents contains scarcely any urease. Our experiments, using the method of the short period incubation, arrived at the same conclusion. In the rumen contents, however, there live numerous micro-organisms and we can not ignore their activities as discussed by Pearson and Smith. We considered it is necessary to study enzymatic activity in relation to the activities of the micro-organisms. In our experiments, therefore,

Omeliansky's medium was added to the reaction medium in order to accelerate the activity of micro- organisms and incubated relatively longer time. According to the results, after 2 days' incubation NH_3 was detected. By this fact, the presence of the urease could be ascertained. In longer incubation, the development of NH_3 increased in the volatile form, but remained constant or decreased in the non-volatile form.

This phenomenon may probably be due to the fact that NH_3 produced in the medium again may have been absorbed by the micro-organisms and transformed into the protein of those in the rumen contents. Thus, although no enzyme could be detected in the short period incubation, it was observable in a longer period incubation. The question whether such phenomenon can be explained by Karstrom's theory[14] regarding enzyme adaptation or not, has yet to be ascertained. But the foregoing reports from our laboratory showed that decomposing power of the cellulose *in vivo* was seven times stronger than *iv vitro*. Therefore, also in the case of urease, it is assumed that this reaction may take place in a shorter time *in vivo* than *in vitro*.

In the case of proteinase also enzymic decomposition occurred increasingly in the course of time, and it may be supposed that the mechanism of the decomposition will be the same as that of urease.

Summary

1. Amylase, urease, proteinase, acid-phosphatase, alkaline-phosphatase and lipase in the rumen contents of goat were determined by the method of short period incubation which is in general use for the enzymatic study. Furthermore, the method of longer period incubation was adopted in the study of enzymes showing negative in a shorter period incubation.

2. In the case of short period incubation, urease, proteinase, lipase were not detected at all, although amylase was found slightly. However, alkaline-phosphatase and acid-phosphatase could be found obviously.

3. In the case of long period incubation the decomposition of urea could be detected after two days' incubation.

4. Ratio of urea decomposition is higher in the solution of the low urea concentration than in that of high one.

5. In the test of urease, volatile NH_3 increased gradually after two days' incubation, neverthless non-volatile NH_3 did not show any increase after 2 days' incubation. Accordingly, the amount of total NH_3 was chiefly due to the volatile NH_3.

6. By the method long period incubation, protein decomposing power of proteinase in the rumen contents could be ascertained.

7. Amylase was detected either in Wohlgemuth's method or the method of

determination of decomposed sugar.

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